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The broad goals of my initial proposal were two-fold. First, I proposed the development of a genetic system that would allow complementation screening in animal cells much in the way that this is done in yeast. Second, I proposed the application of this approach to problems of mortality control and tumorigenesis in breast tumor cells and their normal precursors. As a model system, I chose primary, normal human mammary epithelial cells (HMEC). Progress over the last year can be summarized as follows :

1) Development and validation of the genetic system has been largely completed.
2) We have determined that the lifespan of HMEC cells can be altered by manipulation of telomere length.
3) We have found that a commonly activated cellular oncogene, c-myc, can regulate telomerase activity in HMEC cells and in normal fibroblasts.
4) Either myc expression or telomerase activation can effectively immortalize HMEC cells.
5) We have expanded our genetic analysis of neoplastic transformation to include other aspects of the process, namely resistance to growth inhibitory cytokines.

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FOREWORD

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Introduction

A hallmark of tumor cells is the ability to survive and proliferate under circumstances that are incompatible with the growth and survival of normal cells. Transformation of a normal cell into a tumor cells requires myriad genetic alterations that permit escape from governance by normal growth regulatory programs and that promote unchecked proliferation. Control over the growth of normal cells comes in many forms. For example, the growth and survival of normal cells requires the presence of specific extracellular factors while different factors act in a dominant manner to prevent proliferation. In transformed cells, the dependence on extracellular signals is often reduced by activation of cellular oncogenes; however, in a normal cell, oncogene activation frequently leads to programmed cell death unless additional mutations counter this response. Normal cells are also subject to a pre-programmed limit on the number of divisions that they can execute. After this limit is reached, cells enter replicative senescence, a growth-arrest that is thought to be irreversible. Over the past year, we have used a genetic approach to investigate multiple levels of growth control in mammalian cells. Our efforts have focussed on those that are altered as a normal cell evolves into a tumor cell.

The work supported by this grant has had three specific goals :

1) To develop a set of tools that allow genetic approaches to biological problems in animal cells
2) To identify the genetic events that control telomerase in HMEC cells
3) To identify genetic events that control cellular lifespan in HMEC cells

In the report submitted last year, I described our efforts toward the satisfaction of the first goal. I outlined the retrovirus-based genetic screening system that we have developed and described the deployment of that system in a number of genetic screens. Over the past year, the system has been exploited in several ways. All of our efforts point to the idea that this system will be broadly applicable.

In this year's report, I will focus on results relevant to goals 2 and 3 as outlined above. I also discuss additional work on cytokine resistance that is likely to be relevant to breast cancer.

Body : Results and Discussion

Myc activates telomerase : extension of cellular lifespan.

Our results on the regulation of telomerase and cellular lifespan in HMEC cells were published in Genes and Development (Wang et al. 1998). I have attached a reprint of that manuscript which contains the primary data and a detailed description of the methods. A summary of the results described therein is presented here.

Stabilization of telomeric repeats has been proposed as a prerequisite for tumorigenesis. Circumstantial support for this notion comes from the observation that telomerase is activated in a high percentage of late-stage human tumors, whereas telomerase is largely absent from somatic cells in vivo and from normal human cells in culture. As normal cells proliferate, telomeric repeats are progressively lost due to the incomplete replication of chromosome ends during each division cycle. Telomere shortening has been proposed as the mitotic clock that marks the progress of a cell toward the end of its replicative life-span. According to this model, erosion of chromosome ends triggers cellular senescence. The possibility that telomere maintenance might be an essential component of the tumorigenic phenotype led us to survey known oncogenes for the ability to activate the telomerase enzyme. Of those tested, only c-myc showed a substantial effect on telomerase activity. In fact, expression of myc increased telomerase to a level similar to that seen in a number of breast carcinoma cell lines. Myc could also induce telomerase activity in normal human fibroblast cells, again to levels approximating those seen in fibroblast-derived tumor cell lines.

Recently, a number of subunits of the telomerase enzyme have been cloned (c.f. Harrington et al., 1997; Meyerson,et al., 1997; Nakamura et al., 1997). The abundance of one of these, hEST2 – the catalytic subunit, has been proposed to limit telomerase activity. In fact, expression of hEST2 alone was sufficient to activate the telomerase enzyme in a variety of cell types (Weinrich et al., 1997). In mammary epithelial cells, increased myc expression led to increased abundance of hEST2, providing a possible mechanism for regulation of telomerase activity by myc.

To probe the link between telomere length and replicative lifespan, myc and hEST2 were introduced into normal human mammary epithelial cells which were then continuously cultured. After a defined number of cell divisions (about 22 passages or 50-60 population doublings) normal cells entered replicative senescence. In contrast, cells expressing either myc or hEST2 have continued to proliferate. Escape from senescence was accompanied by either a stabilization or an increase in mean telomere length (for myc and hEST2, respectively). This suggests that the sole act of manipulating telomere length can affect cellular lifespan and establishes telomeres as one mechanism by which cells measure their replicative age.

Promotion of cell proliferation and oncogenic transformation by myc probably requires induction of a number of different target genes. As telomere maintenance may contribute to the long-term proliferative potential of tumor cells, telomerase activation may be an essential component of the ability of myc to facilitate tumor formation.

Immortalization of HMEC.

Since the submission and publication of the data described above, we have continued our analysis of extended-lifepan HMEC cells that have been established following introduction of c-myc or hEST2. We find that these populations are effectively immortal. After more than 150 population doublings beyond the normal senescence point, cells continue to proliferate with no sign of either senescence or crisis. Work over the next year will determine what, if any, additional genetic changes occur as these populations proliferate in culture. This is particularly relevant in light of recent proposals that repair of telomeres can be used to revitalize stem cells from aging patients. Our preliminary findings suggest that as cells with the capacity to maintain telomeres indefinitely (hEST2-expressing, e.g.) are passaged in culture, changes in expression of tumor suppressors occur. A characterization of the precise nature and consequence of these changes is ongoing and will continue through the next year of the granting period.

In addition to our work on HMEC, one other area of investigation relevant to mammary tumorigenesis has developed. This focuses on the regulation of proliferation control by inhibitory cytokines (TGF-β). Although the genetic screen for TGF-β resistance has not been carried out in HMEC cells, the findings are probably relevant to breast cancer.

Resistance to growth inhibition by TGF-β

TGF-β is a multifunctional cytokine that halts the proliferation of many normal epithelial cells. However, tumor cells often acquire resistance to the inhibitory effects of TGF-β (*). To investigate the mechanisms by which cancer cells resist growth inhibitory cytokines, we searched for genes that, when inappropriately expressed, bypass TGF-β-mediated growth arrest. This work led to the identification of three gene products : c-myc, NF-1X and mdm-2. The c-myc oncogene had previously been shown to nullify TGF-β sensitivity in murine keratinocytes, so isolation of this gene in a genetic screen for TGF-β resistance validated our approach. Neither NF-1X nor mdm-2 had previously been connected with the TGF-β response.

The mdm-2 oncogene is a regulator of the p53 tumor suppressor. Increased expression of mdm-2 inhibits p53 function at multiple levels, including antagonism of transcriptional activation and promotion of p53 degradation. Our results therefore seemed to implicated p53 in TGF-β-mediated cell cycle arrest. To test this possibility, we used dominant-negative mutants of p53 to interfere directly with p53 function in our target cells. Surprisingly, we found that loss of p53 function did not affect the ability of TGF-β to enforce growth arrest.

We were therefore left to search for a new mechanism by which the mdm-2 oncogene might affect cell proliferation. Our experiments and those of numerous other labs have suggested that growth arrest following treatment with TGF-β can be eliminated by inactivation of the Rb pathway. Two previous reports had suggested that mdm-2 protein could interact in vitro and in vivo with the Rb protein itself and with a transcription factor, E2F-1, that is regulated by Rb. However, the biological consequences of these interactions were unclear. Following TGF-β treatment, the Rb protein accumulates in the

hypophosphorylated, growth-inhibitory state. In addition, the abundance of E2F-1 protein is diminished, probably through post-translational mechanisms. We found that mdm-2 expression prevented both of these alterations. However, the possibility remained that mdm-2 might accomplish this indirectly by effects on TGF-β signal transduction or through regulation of other cell cycle pathways.

To address more directly the biological interaction between mdm-2 and the Rb pathway, we asked whether mdm-2 expression could abrogate the growth arrest that is enforced by expression of INK4-family CDK inhibitors. INK4 proteins prevent Rb phosphorylation by inhibition of an essential Rb kinase. Rb is therefore locked in a hypophosphylated, growth inhibitory state. One member of the INK4 family, p15 INK4B, is a downstream target of the TGF-β signal transduction pathway and is a likely effector of TGF-β-mediated growth arrest. Expression of mdm-2 substantially rescued the growth inhibition caused by either p15 INK4B or p16 INK4A or, in fact, by enforced expression of Rb itself.

Considered together, our results point to a direct connection between mdm-2 and the Rb pathway. Thus, mdm-2 is a cellular oncogene that, like many viral oncoproteins, can interfere with multiple tumor suppression pathways : the p53 pathway and the Rb/p16 pathway.

The MDM2 oncogene is frequently overexpressed in breast tumors (~70%; c.f. Poremba et. al., 1995; Bueso-Ramos et al., 119; Jiang et al., 1997; Gunther et al., 1997). Furthermore, breast carcinomas are generally resistant to TGF-β while normal mammary epithelial cells are generally sensitive (c.f. Reiss and Barcellos-Hoff, 1997 for review). This led us to ask whether MDM2 expression might confer TGF-β resistance to HMEC cells. Enforced MDM2 expression allowed HMEC cells to form colonies in the continuous presence of TGF-β suggesting that this oncogene might contribute to TGF-β resistance in late stage tumors. In support of this notion, we find that MDM2 expression levels in breast carcinoma cell lines correlates with TGF-β resistance.

I have attached to this report a submitted manuscript containing the relevant data and a detailed description of the methods for the work that has been summarized above.

Conclusions :

The work described in this year's report leads in a number of directions. First, our work on mortality control in HMEC has led to the notion that a human oncogene that is overexpressed in a wide variety of human tumors can regulate telomerase activity. Since we have shown that telomere length can control cellular lifespan, this finding probably explains one aspect of myc-dependent oncogenesis. In addition, the prevalence of myc involvement in human tumors may explain why telomerase is such a broadly expressed marker for late stage cancers. Genetic analysis has also determined that an oncogene, MDM2, that is commonly overexpressed in breast carcinomas can alleviate growth repression by TGF-β in HMEC cells. Late stage and metastatic breast carcinomas secrete large amounts of this growth inhibitory cytokine (Schmid et al., 1995, Reiss et al., 1997; Teti et al., 1997). TGF-β secretion has been proposed to promote metastasis through effects on extracellular matrix. Thus, acquisition of resistance to this cytokine might represent an obligate step on the road to metastatic breast cancer. Another interesting aspect of this work was the conclusion that MDM2 regulates not only the p53 but also the Rb tumor suppressor pathway. Thus MDM2 might contribute to breast carcinoma through multiple routes, acting much like a cellular homolog of SV40 T antigen. In the coming year, further probing into the nature of mortality control in HMEC and into the multiple functions of the MDM2 oncogene may yield new insights into the biology of breast cancer.

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Relevant references are also included with each manuscript provided in the appendix.

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RESEARCH COMMUNICATION

Myc activates telomerase

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Telomere maintenance has been proposed as an essential prerequisite to human tumor development. The telomerase enzyme is itself a marker for tumor cells, but the genetic alterations that activate the enzyme during neoplastic transformation have remained a mystery. Here, we show that Myc induces telomerase in both normal human mammary epithelial cells (HMECs) and normal human diploid fibroblasts. Myc increases expression of hEST2 (hTRT/TP2), the limiting subunit of telomerase, and both Myc and hEST2 can extend the life span of HMECs. The ability of Myc to activate telomerase may contribute to its ability to promote tumor formation.

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Telomerase activity is largely absent from somatic cells in vivo and from normal human cells in culture (Counter et al. 1992). As these cells proliferate, telomeric repeats are progressively lost as a result of incomplete replication of chromosome ends during each division cycle (Watson 1972; Olovnikov 1973; Harley et al. 1990; Hastie et al. 1990). Telomere shortening has been proposed as the mitotic clock that marks the progress of a cell toward the end of its replicative life span. According to this model, erosion of chromosome ends triggers cellular senescence (Harley et al. 1990; for review, see Harley 1991). Bypass of senescence can be accomplished by negation of tumor suppressor pathways (e.g., p53 and Rb/p16). This allows continued proliferation (extended life span) that is accompanied by further telomere loss (Counter et al. 1992). Indefinite proliferation in the absence of a telomere maintenance strategy would eventually result in a complete loss of telomeres and in destabilization of chromosomes (Singer and Gottschling 1994). Because this situation is probably incompatible with survival, cells with an indeterminate life span must adopt strategies for telomere conservation. As predicted, cells that emerge from extended life span as immortal cell lines often activate the telomerase enzyme (Counter et al. 1992; Kim et al. 1994).

Cells that are programmed for continuous proliferation generally maintain telomere length. For example, many stem cell populations possess telomerase activity (Counter et al. 1992; Kim et al. 1994). Telomerase is also induced in mitogen-stimulated lymphocytes and is detected in mitotically active regions of hair follicles and intestinal crypts (for review, see Greider 1998). The association of telomerase with cell proliferation has led to the hypothesis that telomere maintenance is simply a housekeeping function. However, proliferating normal cells in culture generally lack telomerase activity (Counter et al. 1992; Kim et al. 1994).

Stabilization of telomeric repeats may be a prerequisite for tumorigenesis (Counter et al. 1992). Consistent with this notion, telomerase is activated in a high percentage of late-stage human tumors and is present in most tumor-derived cell lines in culture (Counter et al. 1992, 1994; Kim et al. 1994; Shay and Wright 1996). To test the role of telomere maintenance in tumorigenesis, we surveyed known oncogenes for their ability to activate telomerase.

Results and Discussion

Myc activates telomerase

Normal human mammary epithelial cells (HMECs) lack telomerase, whereas immortal HMEC-derivatives and breast tumor cell lines are almost universally telomerase-positive (Shay et al. 1995; Bryan and Reddel 1997; Shay and Bacchetti 1997). Introduction of HPV-16 E6 protein into primary HMECs stimulates telomerase activity, suggesting that, in these cells, a single genetic event can potentiate the enzyme (Shay et al. 1993; Klingelhutz et al. 1996; Fig. 1A,C and 2). Therefore, we asked whether increased expression of other cellular or viral oncogenes could induce telomerase in HMECs. Ectopic expression of *mdm-2* failed to induce telomerase, consistent with the observation that activation of telomerase by E6 is separable from the ability of E6 to promote the degradation of p53 (Klingelhutz et al. 1996; Fig. 2). Several other cellular and viral oncoproteins, including E7, activated Ras (V12), cyclin D1, cdc25C, and cdc25A, also failed to induce telomerase (Fig 2). However, introduction of a c-Myc expression cassette stimulated telomerase activity in HMECs (Figs. 1A and 2). Enzyme activity was elevated within one passage after transduction of HMECs with a retrovirus that directs Myc expression (Fig. 1C). The Myc-expressing populations displayed levels of telomerase activity that approximated those seen in breast carcinoma cell lines (Fig. 1A; e.g., T47D).

Introduction of E6 into normal human diploid fibroblasts failed to activate telomerase (Klingelhutz et al. 1996; Shay et al. 1993; Figs. 1B,C and 2). Similar results were observed for E1A (Fig. 2), activated Ras (V12, not shown), or a dominant-negative p53 allele (Fig. 2). However, telomerase was induced by transduction of either IMR-90 (Figs. 1B,C and 2) or WI-38 cells (Fig. 1C) with a retrovirus that directs c-Myc expression. As with HMECs, activity was apparent immediately after drug

[*Key Words:* Myc; telomerase; hEST2; tumorigenesis]
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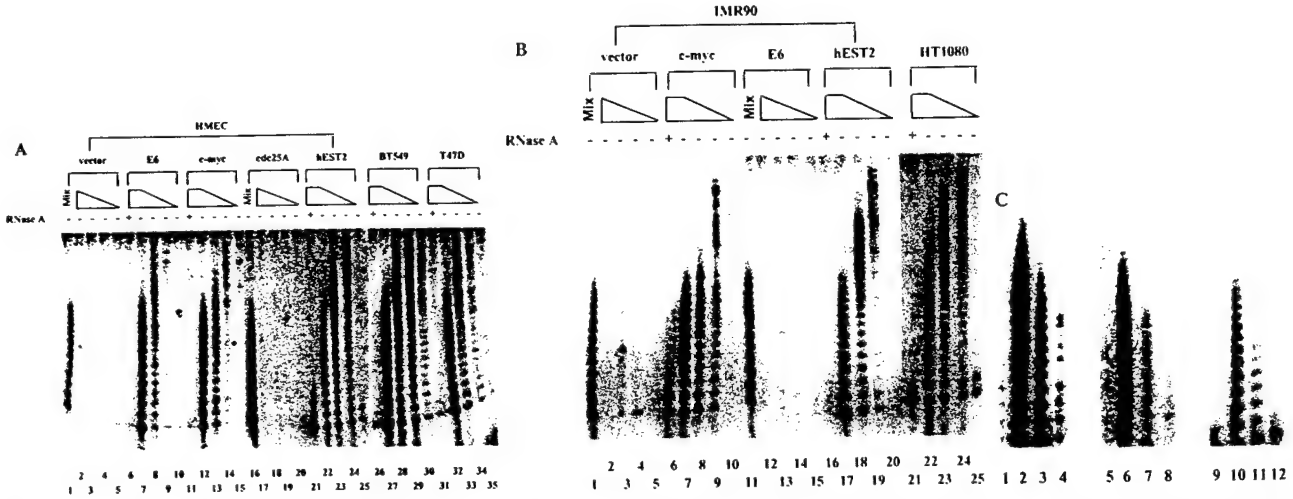

Figure 1. Myc activates telomerase. (*A*) Primary HMECs at passage 12 were infected with empty vector (lanes *1–5*), E6 (lanes *6–10*), c-Myc (lanes *11–15*), cdc25A (lanes *16–20*), or hEST2 (lanes *21–25*) viruses. Breast cancer cell lines BT549 (lanes *26–30*) and T47D (lanes *31–35*) were included for comparison. TRAP assays contained lysates from 10,000 (lanes *2,6,7,11,12,17,21,22,26,27,31,32*), 1000 (lanes *3,8,13,18,23,28,33*), 100 (lanes *4,9,14,19,24,29,34*), or 10 (lanes *5,10,15,20,25,30,35*) cells. (– and +) Absence or presence of RNase A, respectively. (Mix; lanes *1,16*) To exclude the presence of inhibitors in apparently negative lysates, lysate from 10,000 of the indicated cells was mixed with lysate from 10,000 c-Myc-expressing cells. (*B*) IMR90 cells at passage 14 were infected with empty vector (lanes *1–5*), c-Myc (lanes *6–10*), E6 (lanes *11–15*), or hEST2 (lanes *16–20*) viruses. HT1080 cells (lanes *21–25*) were included for comparison. TRAP assays were performed with decreasing cell equivalents as in *A*. (*C*) HMEC (lanes *1–4*), IMR90 (lanes *5–8*), or WI38 (lanes *9–12*) cells were infected with empty vector (lanes *1,5,9*), hEST2 (lanes *2,6,10*), c-Myc (lanes *3,7,11*), or E6 viruses (lanes *4,8,12*). Cells were selected for ~5 days with puromycin or hygromycin and then lysed for telomerase assay. Each lane corresponds to 10,000 cells.

selection (Fig. 1C). The Myc-expressing cells contained levels of telomerase activity comparable to those seen in a telomerase-positive fibrosarcoma cell line, HT1080 (Fig. 1B).

Although c-Myc expression elevates telomerase in both normal epithelial cells and in normal fibroblasts, the HPV-16 E6 protein has been shown to affect telomerase only in epithelial cells (Klingelhutz et al. 1996). Therefore, we questioned the basis of cell-type specific telomerase activation by E6. A recent report suggesting that E6 can activate the Myc promoter (Kinoshita et al. 1997) prompted us to ask whether E6 might regulate telomerase through an effect on Myc expression. In HMECs, expression of E6 induced Myc to levels approaching those achieved upon transduction of HMECs with a Myc retrovirus (Fig. 3A). Surprisingly, E6-induced alterations in Myc protein did not reflect changes in the abundance of *myc* mRNA (Fig. 3B). Therefore, Myc expression must be controlled post-transcriptionally by E6 in HMECs. In contrast, Myc levels remained unaltered following expression of E6 in IMR-90 cells wherein E6 is incapable of activating telomerase (Fig. 3A). Although E6

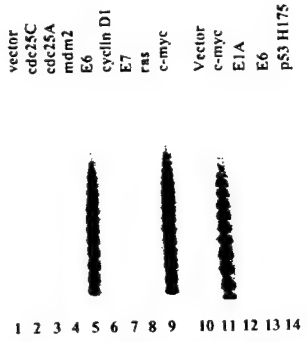

Figure 2. Oncogene activation of telomerase. HMECs (lanes *1–9*) or IMR90 cells (lanes *10–14*) were infected with viruses that direct the expression of the indicated oncogenes (lanes *2–9,11–14*) or empty vector (lanes *1,10*). Cell extracts were analyzed by TRAP assay.

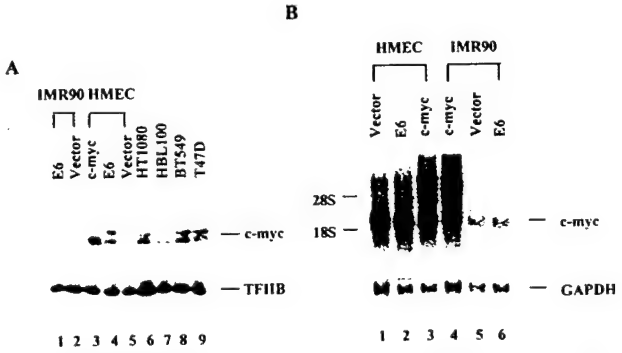

Figure 3. E6 increases c-Myc protein in HMECs. (*A*) Cell lysates from E6 (lane *1*)- and vector (lane *2*)-infected IMR90 cells and lysates from c-Myc (lane *3*)-, E6 (lane *4*)-, and vector (lane *5*)-infected HMECs were analyzed by Western blot with a polyclonal Myc antibody. Tumor cell lines, HT1080 (lane *6*), HBL100 (lane *7*), BT549 (lane *8*), and T47D (lane *9*), were included for comparison. The expression of TFIIB was used to normalize loading. (*B*) Northern analysis of Myc RNA levels in total RNA. GAPDH was probed as a loading control.

may regulate telomerase by other mechanisms, this result is consistent with a model in which E6 regulates telomerase in HMECs by altering the abundance of Myc.

Myc induces hEST2

The presence of the mRNA encoding hEST2, the catalytic subunit of telomerase, strictly correlates with telomerase activity. The hEST2 message is undetectable in normal tissue and in normal cell lines but is present in immortal and tumor-derived cell lines (Harrington et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997). Moreover, hEST2 expression and telomerase are suppressed concomitantly when cells are induced to differentiate (Meyerson et al. 1997). These results suggest that availability of hEST2 may limit telomerase activity, as was demonstrated recently in a number of normal cell lines (Weinrich et al. 1997; Bodnar et al. 1998). Expression of hEST2 could also induce telomerase in HMECs and WI38 and IMR-90 cells (Fig. 1A–C). Activity was apparent immediately following selection of hEST2-expressing cells (Fig. 1C), and the level of telomerase activity observed in hEST2-expressing populations consistently exceeded that observed in cell populations containing c-Myc (Fig. 1A–C).

Because increased expression of hEST2 was sufficient to activate telomerase in both HMECs and in IMR-90 cells (Fig. 1), we asked whether Myc activates telomerase through an effect on hEST2. As expected, hEST2 mRNA was not detectable in normal HMECs, but was induced at least 50-fold following transduction with a Myc retrovirus (Fig. 4A). Thus, Myc regulates telomerase by controlling the expression of a limiting telomerase subunit. Because Myc enhances the expression of responsive genes, its action on hEST2 could be either direct or indirect.

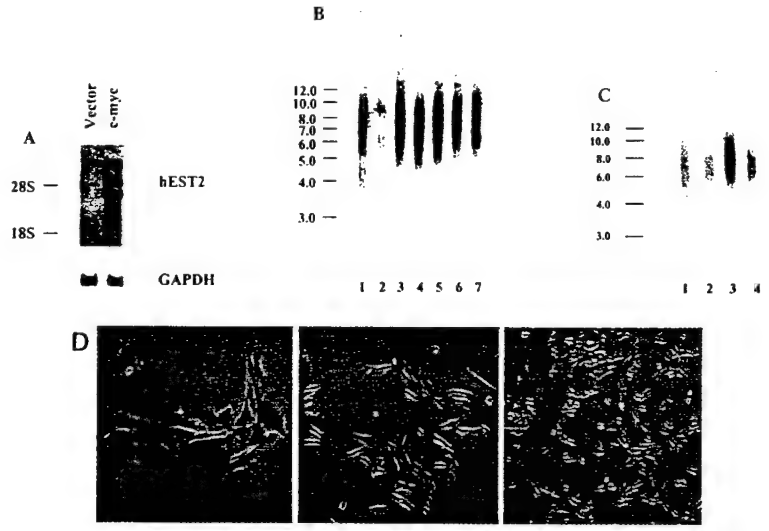

Figure 4. Myc regulates hEST2 and extends cellular life span in HMECs. (*A*) hEST2 Northern analysis of poly(A)$^+$ RNA from normal HMECs and from HMECs that had been infected with a Myc retrovirus. A Northern blot with GAPDH was performed as a loading control. (*B*) Genomic DNA (3 μg) from early-passage HMECs (passage 12, lane *1*), late-passage HMECs (passage 26, lane *2*), and hEST2-expressing HMECs [infected at passage 12 and cultured for 3 (lane *3*), 6 (lane *4*), 8 (lane *5*), 10 (lane *6*), or 14 (lane *7*) additional passages] was digested with *Rsa*I and *Hin*fI. Fragments were separated on a 0.8% agarose gel, and telomeric restriction fragments were visualized with a ^{32}P-labeled human telomeric sequence $(TTAGGG)_3$ probe. (*C*) Genomic DNA (3 μg) from early-passage HMECs (passage 12, lane *1*), vector-infected HMEC (infected at passage 12 and cultured for six additional passages or ~12–14 PD, lane *2*), hEST2-expressing HMECs (infected at passage 12 and cultured for six additional passages or ~12–14 PD, lane *3*), and Myc-expressing cells (infected at passage 12 and cultured for six additional passages or ~18 PD, lane *4*) were digested with *Rsa*I and *Hin*fI. Fragments were probed with a telomeric probe as described in *B*. TRF intensity was quantitated on a Fuji BAS2000 PhosphorImager. Normalizing vector-containing HMECs (lane *2*) to 100 units of intensity, both early passage HMECs (lane *1*) and Myc-expressing HMECs (lane *4*) gave ~150 units of intensity and hEST2-expressing HMECs (lane *3*) gave ~200 units of intensity. (*D*) HMECs transduced with empty vector (*left*), hEST2 (*middle*), or c-Myc viruses (*right*) were grown to a PDL of ~56–60. At this PDL, vector cells adopted a senescent morphology and ceased growth. Cells expressing c-Myc and hEST2 continued to proliferate. To assess the percentage of senescent cells in the population, each culture was stained for senescence-associated β-galactosidase. Greater than 95% of the vector-containing cells were β-galactosidase positive whereas <10% of cells expressing hEST2 or Myc were stained.

hEST2 increases replicative life span in HMECs but not in IMR-90 cells

Preservation of telomeric repeats requires either the telomerase enzyme or the activation of an alternative pathway for telomere maintenance (Kim et al. 1994; Broccoli et al. 1995; Strahl and Blackburn 1996; Wright et al. 1996; Bryan and Reddel 1997). In addition, telomere length can be controlled by telomere-binding proteins (van Steensel and de Lange 1997). To determine whether activation of telomerase in HMECs is sufficient to stabilize telomere length, we followed telomeric restriction fragment (TRF) size as HMECs proliferated either in the presence or absence of telomerase activity. In normal HMECs, telomere length and the abundance of telomeric sequences diminished slightly as cells underwent multiple rounds of division (Fig. 4B). Activation of telomerase by expression of hEST2 not only prevented telomere shrinkage but also increased both the overall abundance of telomeric sequences and the average length of telomeres (Fig. 4B). In Myc-expressing cells, however, the abundance of telomeric sequences was intermediate between that observed in cells expressing hEST2 and that observed in control cells (Fig. 4C). Telomere lengths followed a similar pattern (Fig. 4C).

Generally, in tumors and in immortal cell lines, telomeres are short but stable (Hastie et al. 1990). Comparison of TRF levels in Myc-expressing HMECs to those in early-passage HMECs suggested that Myc probably stabilized telomeres rather than promoted an increase in TRFs as occurs in hEST2-expressing

HMECs (Fig. 4C). Thus, alterations in telomere dynamics after Myc transduction mimic the situation in tumors. Telomerase was ~10-fold more active in hEST2-expressing cells than in Myc-expressing cells. Thus, differences in telomerase activity likely reflect differences in hEST2 expression as the abundance of viral hEST2 mRNA greatly exceeded native hEST2 mRNA levels present in either Myc-expressing HMECs or in any of the tumor cell lines tested to date.

Telomere length has been proposed as the counting mechanism that determines the replicative life span of a cell (Harley 1990; Harley et al. 1991). At a population doubling level (PDL) of ~55–60, vector-containing HMECs ceased proliferation, adopted a senescent morphology (for review, see Stein and Dulic 1995), and stained positive for senescence-associated β-galactosidase (Dimri et al. 1995; Fig. 4D). These cells also showed increased expression of PAI, another senescence marker (Goldstein et al. 1994; data not shown). In contrast, normal HMECs that had received either hEST2 or c-Myc at early passage displayed an extended life span. Cells expressing either c-Myc or hEST2 continued to proliferate beyond the normal senescence point and did not show evident β-galactosidase staining or increased PAI expression (Fig. 4D; data not shown). In both c-Myc and hEST2-expressing cell populations, <10% of cells showed any senescence-associated phenotype at the point at which vector-infected cells senesced. Furthermore, neither population has shown any accumulation of senescent cells during subsequent growth. At present, hEST2- and Myc-expressing populations are a minimum of 40 population doublings (PD) beyond the normal senescence point.

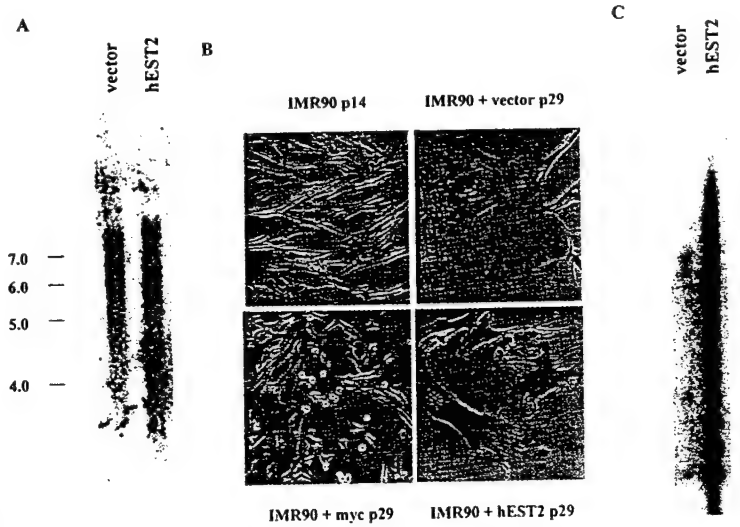

Figure 5. Telomerase activation does not affect life span in IMR-90 cells. (*A*) TRF length of senescent vector-containing IMR-90 and hEST2-expressing IMR-90 cells was analyzed as in Fig. 4. (*B*) Early-passage IMR-90 cells (passage 14) were infected with empty vector, a hEST2 retrovirus, or a Myc retrovirus as indicated. Cells were passaged until the vector-infected cells reached senescence (~15 additional passages). At this time, hEST2 cells also senesced, but Myc-expressing IMR-90 cells continued to proliferate. Shown are senescence-associated β-galactosidase stains of early-passage IMR-90 cells, senescent vector-containing IMR-90 cells, senescent hEST2-expressing IMR-90 cells and Myc-expressing IMR-90 cells that have bypassed the senescence point and entered extended life span. (*C*) Telomerase assays of lysates derived from senescent vector-containing IMR-90 and hEST2-expressing IMR-90 populations. Each lane corresponds to 10,000 cells.

In IMR-90 cells, the consequences of telomerase activation differed from those observed in HMECs. Although hEST2 induced telomerase activity to high levels in early-passage (p14) IMR-90 cells (Fig. 1B,C), this activity was not accompanied by an increase in either the abundance or the length of telomeric sequences (Fig. 5A). To examine the consequences of telomerase activation in normal fibroblasts, vector-containing IMR-90 cells and telomerase-positive hEST2-expressing IMR-90 cells were cultured continuously until the replicative life span of the normal IMR-90 cells was exhausted. Consistent with the idea that telomeres but not telomerase activity per se regulate replicative senescence, hEST2 expression failed to alter the life span of IMR-90 cells. These cells entered replicative senescence within two passages of the point at which normal IMR-90 cells senesced (Fig. 5B). Even after >1 month of maintenance, not a single cell from a population of $>10^6$ cells escaped the senescence block. Senescence was not attributable to a loss of telomerase as the arrested hEST2-expressing IMR-90 population maintained activity (Fig. 5C).

In contrast, IMR-90 cells engineered to express c-Myc display an extended life span (Fig. 5B), even though these cells do not show an obvious stabilization of telomeres. At present Myc-expressing IMR-90 populations have grown for >17 passages (~68 PD) beyond the normal senescence point. Therefore, Myc can extend the life span of a cell even when telomerase activation fails to do so.

These results indicate that the ability of telomerase to extend life span is not universal. Telomerase-positive cells may senesce and still maintain telomerase activity. The mechanisms that regulate the ability of telomerase to extend telomeres and life span may provide an additional level of control over indefinite proliferation and thus tumorigenesis. Furthermore, telomerase-negative cells may adopt alternative strategies for telomere maintenance [alternative lengthening of telomeres (ALT); Bryan and Reddel 1997] and, therefore, achieve immortality without activation of telomerase. The long-term strategy for telomere maintenance adopted by an individual cell will likely depend on the constellation of genetic alterations that such a cell acquires along the pathway to immortality and possibly neoplastic transformation.

The *myc* oncogene is activated by overexpression, gene amplification, translocation, and possibly mutation in a wide variety of different tumor types (Alitalo et al. 1987). Because Myc can elevate telomerase in normal epithelial and fibroblast cells to a level approximating that observed in tumor cell lines, increased Myc activity could account for the presence of telomerase in many

late-stage tumors. In this regard, a study of 100 neuroblastomas revealed that ~20% (16/100) had exceptionally high telomerase activity. Of these, 11 showed amplification of the N-Myc locus (Hiyama et al. 1995). Thus, in this case, telomerase levels correlated well with Myc activation. Although the *myc* oncogene may induce telomerase in a significant proportion of tumors, telomerase may also be regulated by other pathways that contribute to transformation (Holt et al. 1997).

Although telomerase activation has been suggested to be a housekeeping component of a variety proliferative programs (Greider 1998), oncogenic transformation is often achieved through constitutive activation of elements of normal growth control. In this regard, Myc expression accompanies the proliferation of diverse cell types in vivo, and there is significant overlap between contexts in which Myc is expressed and contexts in which telomerase is detected in normal cells. For example, mitogenic stimulation of normal lymphocytes increases Myc levels (Lacy et al. 1986; Kelly and Siebenlist 1988), and stimulated lymphocytes express telomerase (for review, see Greider 1998). Telomerase activity and Myc are also found in human endometrial tissues during the menstrual cycle. Coincidentally, both Myc and telomerase are high during the proliferative phase but are low during the secretory phase (Odom et al. 1989; Kyo et al. 1997). Conversely, Myc is lost as proliferating cells differentiate and exit the cell cycle (e.g., HL-60; Mitchell et al. 1992). Differentiation of these same cells results in loss of both hEST2 expression and telomerase (Meyerson et al. 1997).

The results presented here, considered together with the overlap between Myc activation and telomerase expression in normal tissues, suggest a model in which telomerase may respond to Myc both during the execution of normal proliferation programs and in tumors. Promotion of cell proliferation and oncogenic transformation by Myc probably requires induction of a number of different target genes (for review, see Grandori and Eisenman 1997). In fact, we show that Myc can bypass replicative senescence under circumstances in which telomerase activation alone is ineffective. Thus, telomerase activity in tumors may simply reflect activation of oncogenes such as Myc. However, it is likely that telomere maintenance contributes to the long-term proliferative potential of tumor cells, and therefore telomerase activation may be one component of the ability of Myc to facilitate tumor formation.

Materials and methods

Retroviral plasmids

The following viral plasmids were used: pBabe-puro (Morgenstern and Land 1990), MarXII-hygro, mouse c-myc/MarXII-hygro, mdm-2/MarXII-hygro (from Dr. P. Sun, CSHL), E6/pBabe-puro, cdc25A/MarXII-hygro, cyclin D1/pBabe-puro, rasV12/pBabe-puro, E1A/pWzl-hygro, p53175H/pWzl-hygro, cdc25C/pBabe-puro, and E7/pBabe-puro. The full-length hEST2 cDNA (from Dr. R. Weinberg, MIT, Cambridge, MA) was cloned into pBabe-puro vector at the *Eco*RI and *Sal*I sites.

Cell culture and retroviral-mediated gene transfer

HMEC 184 spiral K cells were from Dr. M. Stampter (Lawrence Berkeley Laboratory, Berkeley, CA); IMR90 and W138 and human breast cancer cell lines BT549, T47D, and HBL100 were from ATCC; and HT1080 cells were from G. Stark (Cleveland Clinic Foundation, OH). The amphotropic packaging line, LinX-A, was produced in our laboratory (L.Y. Xie, D. Beach, and G. Hannon, unpubl.). HMEC were cultured in complete mammary epithelium growth medium (MEGM) (Clonetics). Fibroblasts and LinX-A cells were maintained in DMEM (GIBCO-BRL) plus 10% FBS (Sigma). BT549, HBL100, and T47D were maintained as directed by the supplier. LinX-A cells were transfected by calcium-phosphate precipitation with a mixture containing 15 μg of retroviral plasmid and 15 μg of sonicated salmon sperm DNA. Transfected cells were incubated at 37°C for 24 hr and then shifted to 30°C for virus production. After 48 hr, virus was collected, and the virus-containing medium was filtered to remove packaging cells (0.45-μm filter; Millipore). Target cells were infected with virus supernatants supplemented with 4 μg/ml polybrene (Sigma) by centrifugation for 1 hr at 1000*g* and incubation at 30°C overnight. Infected cells were selected 48 hr after infection with the appropriate drugs (hygromycin, G418, or puromycin).

Telomerase assays and expression analyses

The TRAP assay was performed essentially as described (Kim et al. 1994) with some modification. Briefly, extracts were prepared in lysis buffer (10 mM Tris at pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol) and cleared by centrifugation for 30 min at 50,000*g*. Lysate corresponding to from 10 to 10^4 cells was used. Telomeric repeats were synthesized onto an oligonucleotide, TS (5′-AATCCGTCGAGCAGAGTT-3′), in an extension reaction that proceeded at 30°C for 1 hr. Extension products were amplified by PCR in the presence of [^{32}P]dATP with TS and a downstream anchor primer (5′-GCGCGGCTAACCCTAACCCTAACC-3′). Five microliters of each reaction was analyzed on a 6% acrylamide/8 M urea gel.

TRF length was measured as described by Strahl and Blackburn (1996) and senescence-associated β-galactosidase activity was determined as described by Serrano et al. (1997).

For Northern blotting, total RNA was isolated from subconfluent cultures by use of Trizol reagent (GIBCO-BRL). Total RNA (10 μg) or poly(A)$^+$ RNA (5 μg) was resolved by electrophoresis and transferred to Hybond-N$^+$ membranes according to the manufacturer's instructions. hEST2 was visualized after hybridization with a labeled *Stu*I fragment of hEST2 (Meyerson et al. 1997). Myc and GAPDH were visualized with probes derived from cDNAs.

Western blotting was performed essentially as described by Harlow and Lane (1988). Cells were washed with cold PBS and lysed in Laemmli loading buffer. Lysates were heated at 95°C for 10 min. Samples were separated on 8% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated with either a c-Myc rabbit polyclonal antibody (N-262; Santa Crutz) or a TFIIB rabbit polyclonal antibody (from Dr. B. Tansey, CSHL). Immune complexes were visualized by secondary incubation with ^{125}I-labeled protein A (ICN).

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p53-Independent Function of MDM2 may Contribute to TGF-β Resistance in Tumors

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TGF-β inhibits the proliferation of cells of epithelial, lymphoid and neuroectodermal origins, and loss of TGF-β-sensitivity has been linked to tumorigenesis. Since the mechanisms of TGF-β–resistance are not well-understood, we performed a genetic screen to search for cDNAs that could abrogate the TGF-β-sensitivity. We found that ectopic expression of MDM2 rescued TGF-β -induced growth arrest. This was not achieved by inactivation of p53 but instead by interference with Rb/E2F function. In human breast tumor cell lines, increased levels of MDM2 expression correlated with TGF-β resistance. Thus MDM2 may confer TGF-β resistance to tumors wherein MDM2 is often overexpressed. Furthermore, MDM2 may promote tumorigenesis by interference with two independent tumor suppressors, p53 and Rb.

The TGF-β signaling pathway has been implicated in suppressing tumorigensis in animals. Loss of TGF-β-sensitivity is frequently observed in tumors derived from cells which are normally sensitive. In human breast carcinomas, colon carcinomas and melanomas, the extent of TGF-β-resistance correlates with malignancy (1). Furthermore, inactivation of TGF-β signaling by a homologous null mutation in *Smad3* gene leads to the development of metastatic colorectal adenocarcinomas in mice (2). Therefore, understanding the molecular events that confer TGF-β-resistance in tumors may shed light on general mechanisms underlying neoplastic transformation and malignant progression. Currently, the mechanism

of TGF-β-resistance in tumor cells is not well-understood. Previous studies suggested that some tumors may have become TGF-β-resistant due to loss-of-function mutations in genes encoding type II TGF-β receptor (3, 4), Smad2 (5) or Smad4 (6) which are essential components in the TGF-β signaling pathway or through deletion of chromosome 9p21 which contains the $p15^{INK4B}$ locus (7). However, these alterations cannot account for the majority of cases in which TGF-β-responsiveness is lost. Therefore, TGF-β–resistance in tumors must also be achieved by mechanisms that are currently unknown.

In an attempt to identify alterations that could lead to TGF-β–resistance in tumor cells, we took a genetic approach. We systematically screened for genes which could, upon overexpression, allow cells to escape TGF-β-induced growth arrest (Fig.1A and ref. 54). A cDNA library was introduced into Mv1Lu, a TGF-β–sensitive cell line, using a retroviral gene transfer system (55). Infected cells were grown in the presence of TGF-β in order to select for TGF-β-resistance. To minimize background, cells infected with the library were selected in TGF-β with frequent TGF-β and medium replenishment for a period of three months, by which time the majority of naturally-occurring resistant cells had been eliminated (8, 9). Proviruses were then recovered from cells that could proliferate in the presence of TGF-β. The screen yielded three genes, *Mdm2*, *c-myc* and *Nfix-1*, that were capable of conferring TGF-β–resistance (Fig. 1B, top panel). While *c-myc* was represented by one clone, both *Mdm2* and *Nfix-1* were each independently isolated several times. When treated with TGF-β, cells expressing MDM2, *c-*

myc or NFIX-1 formed colonies and exhibited a morphology that was identical to the morphology of non-treated cells. By contrast, control cells (either non-infected cells or those infected with a control virus) did not form colonies and showed a flattened morphology characteristic of arrested cells (Fig 1B and data not shown).

c-myc overexpression has previously been demonstrated to overcome TGF-β arrest in mouse keratinocytes (10). Thus, the isolation of *c-myc* validated the screen and confirmed the ability of *c-myc* to overcome TGF-β-induced growth arrest in epithelial cells.

NFIX-1 is a member of a family of transcription factors which may function in development and cell differentiation (11). Many TGF-β-responsive promoters contain binding sites for NFI family members (12, 13, 14), and TGFβ is able to stimulate the activity of NFIC, another member of this family (15). At present the exact mechanism by which NFIX-1 confers TGF-β-resistance remains to be investigated.

MDM2 is an oncogenic protein whose expression is frequently increased in a broad spectrum of tumors (16). However, the ability of MDM2 to interfere with inhibitory cytokines had not previously been suspected. Therefore, we concentrated on understanding the mechanisms by which MDM2 confers TGF-β–resistance.

We reasoned that TGF-β–resistance could be achieved by interference either with the TGF-β signaling pathway or with events

downstream of the signaling pathway (e.g., those that enforce growth arrest). Therefore, we asked at which of these two levels MDM2 exerted its effect. Activation of TGF-β signaling is known to alter expression of a battery of genes. For example, TGF-β induces expression of both plasminogen activator inhibitor-1 (PAI-1) and $p15^{INK4B}$ (17, 18) but represses the expression of *c-myc* (19) and cdc25A (20). In MDM2-expressing cells, PAI-1 and p15 genes were each induced by TGF-β treatment (Fig. 1C). Likewise, MDM2 failed to rescue the suppression of *c-myc* and cdc25A (data not shown). These results suggested that TGF-β signaling was still intact in MDM2-expressing cells and that MDM2 must mediate TGF-β–resistance through bypass of TGF-β responses.

MDM2 was originally characterized as a protein that associates with tumor suppressor protein p53 (21). The binding of MDM2 to p53 inhibits p53-mediated transcription by masking the activation domain of p53 (22, 23) and also promotes proteasome-dependent p53 degradation (24, 25). Therefore, we asked whether MDM2 bypassed TGF-β-induced growth arrest through elimination of p53 function. Mv1Lu cells contain wild-type p53 activity (26). To test the possibility that MDM2 bypassed TGF-β induced growth arrest through an effect on p53 protein, we interfered with endogenous p53 by introducing into Mv1Lu cells two dominant-negative p53 alleles, p53Val135 or p53-175H. p53Val135 is a temperature-sensitive mutant. At the permissive temperature (32 °C), p53Val135 has wild-type activity, and its overexpression leads to cell cycle arrest. At the non-permissive temperature (39 °C), p53Val135 not only is inactive

but also inactivates, in trans, endogenous wild-type p53 (27, 28). p53-175H, which was isolated from a human colon carcinoma, is also able to interfere with wild-type p53 function in a dominant-negative fashion (21, 29). The effect of these p53 mutant proteins on the wild-type p53 in Mv1Lu cells was deduced from their ability to interfere with p53-dependent transcription. Expression of either p53Val135 at 39 °C or p53-175H at 37 °C reduced the transcription from a p53-dependent reporter (PG13-Luc) (30) to almost background levels while having no effect on a p53-independent promoter, TK-Luc (Fig 2A). Also, the growth of Mv1Lu cells expressing p53Val135 mutant was inhibited at 32 °C (Fig. 2B), indicating that the pathway leading to p53-induced growth arrest is intact in these cells. Cells expressing p53Val135 at 39 °C or those expressing p53-175H at 37 °C retained TGF-β-sensitivity (Fig. 2B). Therefore, despite the fact that both the p53 mutants and MDM2 shared the ability to abolish p53 function in Mv1Lu cells, only MDM2 could confer TGF-β-resistance. Furthermore, when the p53-binding domain (amino acids 19-102) was deleted from MDM2, MDM2 was still able to confer TGF-β resistance in Mv1Lu cells (Fig. 2C), despite the fact that it could no longer bind to p53 (Fig. 2D). These results suggested the MDM2-mediated bypass of TGF-β-sensitivity does not depend on its ability to inactivate p53.

It has been well documented that TGF-β induces G1 arrest through the Rb/E2F pathway, and viral oncogenes which interfere with this pathway confer TGF-β–resistance (19, 31) TGF-β treatment leads to a shift of Rb into the hypophosphorylation, growth-inhibitory form. This effect correlates with TGF-β-induced cell cycle

arrest (31). Indeed, expression of HPV-16 E7 protein, which abolishes Rb function but not p53 function (32, 33), was sufficient to establish TGF-β resistance in Mv1Lu cells (Fig. 3D). This led us to investigate the possibility that MDM2-mediated bypass of TGF-β–sensitivity is achieved by interference with RB/E2F functions. This notion was supported by the recent finding that MDM2 can bind Rb and E2F/DP directly (34, 35), thus suggesting that MDM2 may interact functionally with the Rb/E2F pathway.

In wild-type Mv1Lu cells, TGF-β treatment led to a gradual change in Rb phosphorylation status (Fig. 3A). After 24 hours (the time at which growth arrest was established), the majority of Rb had shifted from hyperphosphorylated form to the fast-migrating, growth-inhibitory, hypophosphorylated form. However, in MDM2- and *c-myc*-expressing cells, the majority of Rb remained hyperphosphorylated even 24 hours after TGF-β addition. These results demonstrated that MDM2 could prevent the loss of Rb phosphorylation that was induced by TGF-β. The capacity of MDM2 to keep Rb in its hyperphosphorylated, non-growth-inhibitory state is consistent with its ability to overcome TGF-β-induced growth arrest.

E2F proteins are a family of transcription factors which bind to unphosporylated Rb protein. Upon phosphorylation of Rb, E2F proteins are released and form transcriptionally active complexes with another family of transcription factors, DP. E2F/DP complexes can promote cell growth by regulating the transcription of

downstream genes (36). Therefore, we examined possible effects of MDM2 on E2F/DP activities. Endogenous E2F/DP activities were assessed by measuring the expression of an E2F/DP-dependent luciferase reporter gene (37) that was transiently transfected into Mv1Lu cells. In contrast to previous studies in other cell lines (34, 35), expression of MDM2 did not increase E2F/DP activity in Mv1Lu cells (Fig. 3B). TGF-β treatment reduced E2F/DP activity by two fold in wild-type cells. In MDM2-expressing cells, this reduction was abolished (Fig, 3B). Neither TGF-β nor MDM2 altered the expression of a promoter containing mutant E2F/DP binding sites, indicating that the effects of TGF-β and MDM2 were specific.

Treatment with TGF-β has been demonstrated to reduce E2F-1 mRNA levels in Mv1Lu cells (38). We therefore tested whether alterations of E2F/DP activity by either TGF-β treatment or MDM2 overexpression reflected changes in E2F-1 protein levels. TGF-β treatment led to a gradual decrease in E2F-1 protein levels in Mv1Lu cells. This decrease was prevented by MDM2 expression (Fig. 3C). Based on these observations, we reasoned that restoration of E2F-1 protein levels might contribute to the ability of MDM2 to confer TGF-β–resistance. In accord with this hypothesis, enforced E2F-1 expression in Mv1Lu cells led to TGF-β–resistance (Fig. 3D).

Considered together, our data indicated that one component of the mechanism by which MDM2 rescues TGF-β-induced growth arrest is through restoration of E2F-1 protein levels. MDM2 could prevent down-regulation of E2F-1 expression, or alternatively,

MDM2 could stabilize E2F-1 protein directly. Overexpression of *c-myc* also restored E2F-1 protein levels and E2F/DP activities in the presence of TGF-β (Fig. 3B and 3C), which suggested that *c-myc* could confer TGF-β-resistance through overlapping mechanisms.

MDM2 is frequently overexpressed in human tumors [Momand, 1997 #47]. In this study, we identified the biological consequence of MDM2 overexpression, bypass of TGF-β–induced growth arrest. TGF-β induces growth arrest in normal human lymphocytes, melanocytes, and breast epithelial cells. However, cells from human leukemia, lymphomas, melanomas and breast carcinomas are resistant to TGF-β treatment (39, 40, 41, 42). Coincidentally, MDM2 is also overexpressed in these tumors at a high frequency (e.g., 73% in human breast carcinomas) (43, 44, 45, 46, 47). Enforced expression of MDM2 in primary human mammary epithelial cells (HMEC) converted these normally TGF-β sensitive cells to a TGF-β resistant phenotype (Fig. 1B). Considered together, these observations raised the possibility that MDM2 overexpression may contribute to TGF-β-resistance in tumors.

To investigate a possible role for MDM2 in TGF-β resistance in tumors, we examined MDM2 expression levels (Fig. 4A) and TGF-β responsiveness (Fig. 4B) in 7 human breast tumor cell lines, MCF-7, T-47D, BT549, HBL100, ZR-75-1, MDA-MB-468 and HTB20. T-47D, ZR-75-1 and HTB20 cells expressed MDM2, at levels comparable to those in TGF-β resistant HMEC and Mv1Lu cells transduced with MDM2 retroviral vectors. These three cell lines were completely

resistant to TGF-β induced growth arrest. The two cell lines (MCF-7 and BT549) which were most sensitive to TGF-β treatment showed very low MDM2 levels. The MDM2 expression levels in these two cell lines were similar to levels found in TGF-β sensitive HMEC. Thus increased MDM2 expression strictly correlated with the ability to escape TGF-β induced growth inhibition in these five cell lines. Two other lines (HBL100 and MDA-MB-468) exhibited partial resistance to TGF-β despite low levels of MDM2 expression. Other mechanisms (e.g., *c-myc* overexpression) might be responsible for TGF-β resistance in HBL100 and MDA-MB-468 cells. Our data suggested that in human breast carcinomas MDM2 overexpression may be one genetic alteration which allows tumor cells to become resistant to growth inhibitors such as TGF-β.

In breast carcinomas and melanomas, the extent of MDM2 overexpression correlates with metastatic progression (43, 45). In these tumors increased TGF-β–resistance was also observed in late metastatic stages (41, 42). In our study, T-47D, ZR-75-1 and HTB20, the 3 cell lines which expressed highest levels of MDM2 and exhibited complete TGF-β resistance, were all derived from late-stage, metastatic breast carcinomas. As breast carcinomas and melanomas become metastatic, they secrete large amounts of TGF-β (40, 42) which may enhance tumor cell invasion and metastasis through effects on extracellular matrix (42, 48). Thus MDM2 overexpression in late stage breast carcinomas and melanomas may be a crucial adaptation that is necessary for maintenance of

proliferation in the presence of increased TGF-β levels during the development of tumor invasiveness.

Previous studies indicate that elimination of TGF-β mediated growth inhibition is indeed a critical step in the development of metastatic cancers. For example, a null mutation in *Smad3* gene, an essential component of TGF-β signaling pathway, promotes the formation of metastatic tumors (2). Loss of TGF-β sensitivity in tumors can be achieved through multiple mechanisms (e.g., *c-myc* overexpression, loss of TGF-β receptor function and mutations in *Smad* genes). Here we have demonstrated that MDM2 overexpression, which is frequently observed in tumors, was alone sufficient to confer TGF-β resistance. However, it is possible that multiple mechanisms of cytokine resistance are operating in a given tumor.

Previous studies cataloging genetic alterations in tumors have suggested that MDM2 has functions in addition to inactivation of p53. In human breast carcinomas and lymphomas, p53 mutation and MDM2 overexpression were observed in the same tumors (46, 47) In other studies, tumors harboring both p53 mutation and MDM2 overexpression have worse prognosis or become more invasive than tumors with only one genetic change (49). Recently, several alternatively spliced forms of MDM2 were cloned from bladder and ovarian carcinomas (50). These alternatively spliced forms lack the p53-binding domain but are still able to transform NIH3T3 cells. Similar non-p53-binding MDM2 proteins are overexpressed in

human astrocytic neoplasms (51). Therefore, the inactivation of p53 is not the only route through which MDM2 contributes to neoplastic transformation. Our study demonstrated that MDM2 could bypass the negative growth control by TGF-β, and possibly other growth inhibitors, through interference with the RB/E2F pathway. As predicted by our results, enforced expression of MDM2 could prevent the growth arrest that was induced by expression of INK4 family members (p16, data not shown) which prevent proliferation by a direct effect on the Rb pathway. These observations provide a potential mechanism underlying p53-independent oncogenic activity of MDM2 and indicate that, when overexpressed in tumors, MDM2 could antagonize both p53 and Rb tumor suppressor pathways, functioning like a cellular version of SV40 T antigen.

Figure Legends

Figure 1. Isolation of *Mdm2*, *c-myc* and *Nfix-1* as genes bypassing TGF-β –induced growth arrest in Mv1Lu cells.

A) Schematic outline of the screen designed to identify genes that confer TGF-β resistance when ectopically expressed in Mv1Lu cells.

B) *Top panel*, Mv1Lu cells expressing MDM2, *c-myc* or NFIX-1 were resistant to growth arrest induced by TGF-β. Wild-type Mv1Lu or Mv1Lu expressing *c-myc*, MDM2, or NFIX-1 (as indicated) were treated with indicated concentrations of TGF-β for 8 days. Colonies were visualized by staining with crystal violet. *Bottom panel*, HMEC

cells expressing MDM2 were resistant to growth arrest induced by TGF-β. HMEC cells at passage 14 was infected with a retroviral vector expressing MDM2 and infected cells were selected with hygromycin. Three more passages after infection, HMEC expressing MDM2, together with wild-type HMEC at passage 17, were treated with indicated concentrations of TGF-β for 16 days. Colonies were visualized by staining with crystal violet

C) The expression of PAI-1 and $p15^{INK4B}$ genes were induced by TGF-β in wild-type Mv1Lu cells and Mv1Lu cells expressing MDM2. PolyA+ RNA was isolated from TGF-β treated (+, 5ng/ml for 6 hours) or non-treated (-) wild-type Mv1Lu cells (WT), MDM2-expressing cells (MDM2) or selected pools of TGF-β-resistant clones expressing MDM2 (MDM2 Clns). 2.5 μg of polyA+ RNA was run on denaturing gel and transferred to hybond N+ membrane, which was then hybridized to a mouse p15 gene probe (top panel) or human PAI-1 probe and GAPDH probe (lower panel). The positions of p15, PAI-1 and GAPDH mRNA are indicated with arrows.

Figure 2. MDM2 conferred TGF-β resistance through a p53-independent mechanism in Mv1Lu cells.

A) Expression of p53Val135 and p53-175H mutants blocked the ability of endogenous p53 to activate transcription in Mv1Lu cells. Mv1Lu cells were infected with a recombinant retrovirus encoding p53Val135 (HygroMaRXII-p53Val135) or p53-175H (HygroMaRXII-p53-175H). Infected cells were selected with hygromycin. With the

calcium phosphate co-precipitation method, wild-type Mv1Lu cells (WT) or cells expression p53Val135 (p53Val135) or p53-175H (p53-175H) were transfected with PG13-Luc, a p53-responsive luciferase reporter plasmid (top panel), or TK-Luc, a non-p53-responsive reporter plasmid in which luciferase expression is driven by a thymidine kinase promoter (bottom panel). All transfections included pSV-LacZ which expresses LacZ gene from a SV40 early promoter. Cells were lysed 48 hours after transfection and luciferase and β-galactosidase activities were measured (Promega). Values represent means +/- SEM of luciferase activities (normalized to β–galactosidase activities) from three independent transfections.

B) Mv1Lu cells expressing dominant negative mutants of p53 protein were still sensitive to TGF-β induced growth arrest. Wild-type Mv1Lu cells or cells expressing MDM2, p53Val135, or p53-175H (as indicated) were subjected to TGF-β treatment at indicated concentrations and temperatures for 8 days. Colonies were then stained with crystal violet.

C). MDM2 protein lacking p53 binding domain conferred TGF-β resistance. Wild-type Mv1Lu cells or cells expressing wildtype MDM2 or MDM2 lacking the p53 binding domain (as indicated) were subjected to TGF-β treatment at indicated concentrations for 8 days. Colonies were then stained with crystal violet.

D). MDM2 protein with a deletion from residue 19 to 102 could not bind to p53. Wildtype or mutant MDM2 proteins were translated *in*

vitro from pcDNA3 vectors with the TNT coupled reticulocyte lysate system (Promega) in the presence of ^{35}S-Methionine and incubated with a GST-p53 fusion protein bound to glutathione-Sepharose 4B beads. Proteins bound to beads after washing (the right two lanes), or part of the *in vitro* translation reactions (equal to 1/5 of the input in binding reactions) (the left two lanes), were separated by 12% SDS-PAGE and radio-labeled proteins were visualized by autoradiogram.

Figure 3. MDM2 overexpression interfered with TGF-β induced changes in the Rb/E2F pathway.

A). Expression of MDM2 in MV1Lu cells prevented the TGF-β-induced shift of Rb to the hypophosphorylated form. Wild-type Mv1Lu cells or cells expressing MDM2 or *c-myc* (as indicated) were treated with TGF-β (5 ng/ml) for 0, 2, 12 or 24 hours. Cell lysates were prepared. Proteins were separated by SDS-PAGE and were transferred to a nitrocellulose membrane. The membrane was then probed with an antibody against Rb (G3-245, PharMingen) and immunocomplexes were visualized by ECL. The positions of the bands representing under-phosphorylated Rb (RB) or hyper-phosphorylated Rb (RB^{phos}) are indicated by arrows.

B). MDM2 blocked the down-regulation of E2F/DP activity by TGF-β. Wild-type, MDM2-expressing or *c-myc*-expressing (as indicated) Mv1Lu cells were transfected with a E2F/DP-dependent luciferase reporter plasmid containing 3 copies of an E2F/DP DNA binding site

(E2FWT-Luc, top panel) or a non-E2F-dependent luciferase reporter plasmid containing 3 copies of a mutant E2F DNA binding site (E2FMut-Luc, bottom panel). pSV-LacZ was included as an internal control in all transfections. 24 hours post transfection, cells were either treated with 5 ng/ml TGF-β for 18 hours (TGF-β) or left untreated (No TGF-β). Cells were then lysed and luciferase and β-galactosidase activities were measured (Promega). Values represent means +/- SEM of luciferase activities (normalized to β–galactosidase activities) from three independent transfections.

C). MDM2 prevented the down-regulation of E2F-1 protein level by TGF-β. Wild-type, MDM2-expressing or *c-myc*-expressing (as indicated) Mv1Lu cells were treated with 5 ng/ml TGF-β for 0, 15 or 27 hours. Cell lysates were prepared. Proteins were separated by SDS-PAGE and were transferred to a nitrocellulose membrane. The membrane was then probed with an antibody against E2F-1 (C20, Santa Cruz) and immunocomplexes were visualized by ECL. The position of the band representing E2F-1 protein is indicated with an arrow.

D). Ectopic expression of E2F-1 and HPV16 E7 conferred TGF-β resistance. Mv1Lu cells were infected either with a recombinant retrovirus encoding E2F-1 (pBabe-puro-E2F-1) or with a retrovirus encoding HPV16 E7 (HygroMaRXII-E7). Infected cells were selected with puromycin or hygromycin, respectively. Wild-type, E2F-1-expressing, E7-expressing or MDM2-expressing (as indicated) Mv1Lu

cells were treated with TGF-β at the indicated concentrations for 8 days. Colonies were stained with crystal violet.

Figure 4. Increased MDM2 expression levels correlated with resistance to TGF-β induced growth inhibition in human breast tumor cell lines.

A). MDM2 protein levels in human breast tumor cell lines and wild-type or MDM2-expressing HMEC and Mv1Lu cells. Cell lysates were prepared from human breast tumor cell lines and wild-type or MDM2-expressing HMEC and Mv1Lu cells (as indicated). Proteins were separated by SDS-PAGE and were transferred to a nitrocellulose membrane. The membrane was then probed with an antibody against MDM2 (2A10) and immunocomplexes were visualized by ECL. The position of the band representing MDM2 protein is indicated with an arrow.

B). TGF-β responsiveness of human breast tumor cell lines. The human breast tumor cells were treated with at indicated TGF-β concentrations for 20 days. Colonies were stained with crystal violet.

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54. Since it has been shown that TGF-β promotes growth in Swiss 3T3 and Balb 3T3 cells (52), a mixture of polyA+ RNA from these cell lines was chosen as the source of our cDNA library. The library was cloned into a retroviral expression vector HygroMaRXII

(PS, GJH and DB, unpublished, ref 55), packaged in an ecotropic virus packaging cell line LinX E (Lin Ying Xie, DB and GJH, unpublished), and used to infect Mv1Lu cells which had been engineered to express the ecotropic virus receptor (53). We estimated that a total of 10^7 cells were independently infected by the viral library. The infected cells were selected with hygromycin and then subjected to TGF-β treatment. Previously we observed that about $20/10^6$ of wild type Mv1Lu cells escaped TGF-β arrest under the conditions of our screen. To minimize background, we took advantage of observation that most of the naturally-occurring resistant cells would regain sensitivity after long-term TGF-β treatment (8, 9 and PS unpunlished). Infected cells were maintained in 100 separate plates at a density of $2X10^5$ and were passage independently in TGF-β for 3 month. By this time, cells had disappeared from 80% of parallel control plates which contained cells infected with HygroMaRXII-LacZ virus, while 54 plates infected with the library still contained fast-growing cells. Integrated proviruses were then excised with Cre recombinase from the genomic DNA that was isolated from the library plates and cDNA from 38 plates of resistant cells have been recovered, sequenced and retested thus far. Among these, 7 plates contained cDNA encoding *Mdm2*, 1 contained *c-myc*, and 7 contained *Nfix-1*.

55. The cDNA expression vector (HygroMaRXII) was designed based on Molony murine leukemia virus (MoMLV). We have included a recognition site (loxP) for Cre recombinase in 3' LTR and a bacterial replicon and a bacterial selectable marker within the retroviral genome. These modifications allowed easy and efficient recovery of

cDNAs by Cre-mediated excision of integrated proviruses from the genome. The recovered circular plasmids contained a single LTR, and thus could be directly used to produce recombinant viruses for retests and further studies.

56. To test the ability of various gene to overcome TGF-β-induced growth arrest, Mv1Lu or HMEC cells were infected with recombinant retroviruses encoding these genes in hygroMaRXII or pBabe-puro. The infected cells were selected with hygromycin (200 μg/ml for Mv1Lu or 5 μg/ml for HMEC) or puromycin (1.5 μg/ml), seeded on 6-well plates (4000 cells/well) and subjected to TGF-β treatment at concentrations of 0, 2.5, 5, 10, 20 and 50 ng/ml. Medium was replaced every 4 days and cells were stained with crystal violet after 8 (Mv1Lu) or 16 (HMEC) days of TGF-β treatment.

57. Western blotting analysis were performed to examine the phosphorylation status of Rb, E2F-1 expression levels in various Mv1Lu cells and MDM2 expression levels in human breast tumor cell lines. Cells were treated with 5 ng/ml TGF-β for an indicated period of time, if needed, before lysed in a buffer containing 50 mM Tris pH8.0, 120 mM NaCl, 0.5% NP-40 and 'complete' protease inhibitor mix (Boeheringer). 100 μg of each cell lysate was separated on a 12% or 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then probed with an antibody against Rb (G3-245, PharMingen), E2F-1 (C20, Santa Cruz) or MDM2 (2A10, a gift from Drs. Arnold Levine and Martine Roussel) and immunocomplexes were visualized by ECL.

58. We thank Dr. B. Vogelstein for providing PG13-Luc, Dr. D. Livingston for providing pBabe-puro-E2F-1 and E2F/DP reporter

plasmids, Drs. Arnold Levine and Martine Roussel for providing anti-MDM2 antibody 2A10, and Drs. Roberta Maestro and Doug Conklin for helpful comments on the manuscript. TGF-β is a kind gift of Berlex, Inc. (Richmond Ca.). Supported in part by grants from NIH (DB and GJH) and the US Army (GJH). PS is a postdoctoral fellow of Helen Hay Whitney Foundation. GJH is a Pew Scholar in the Biomedical Sciences. DB is the Hugh and Catherine Stevenson Professor.

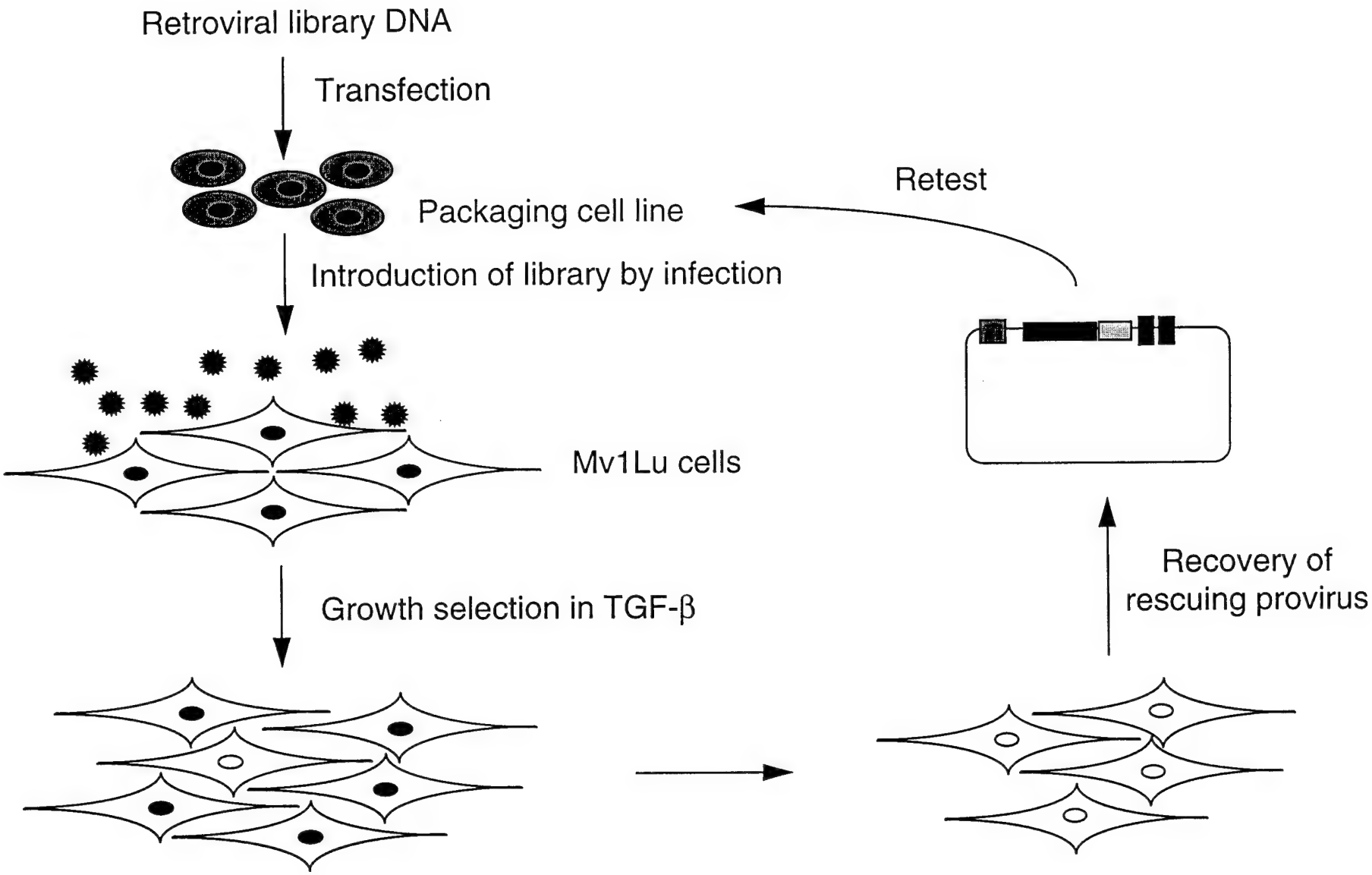

Retroviral library DNA
Transfection
Packaging cell line
Retest
Introduction of library by infection
Mv1Lu cells
Growth selection in TGF-β
Recovery of
rescuing provirus

Figure 1A

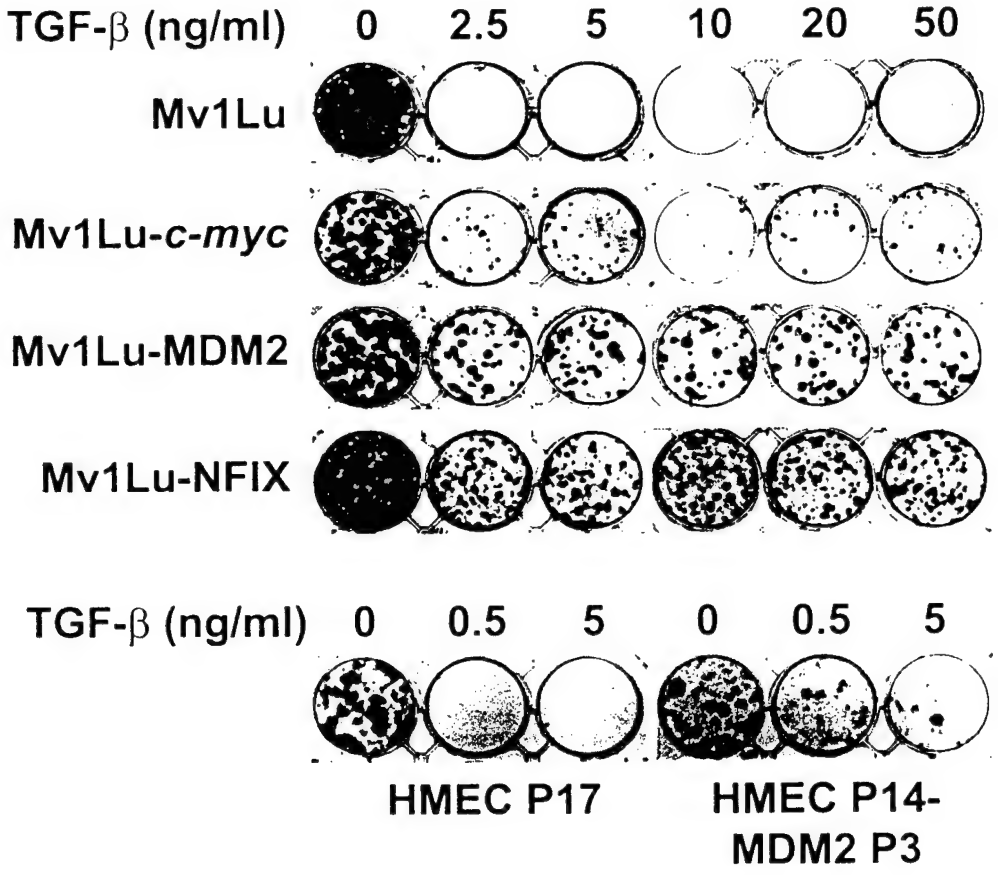

TGF-β (ng/ml) 0 2.5 5 10 20 50
Mv1Lu
Mv1Lu-c-myc
Mv1Lu-MDM2
Mv1Lu-NFIX
TGF-β (ng/ml) 0 0.5 5 0 0.5 5
HMEC P17
HMEC P14-MDM2 P3

Figure 1B

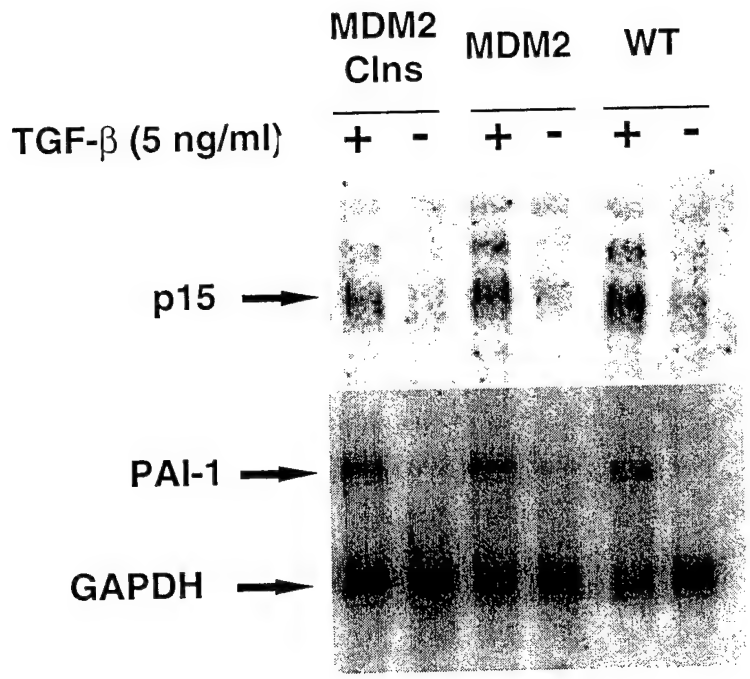


Figure 1C

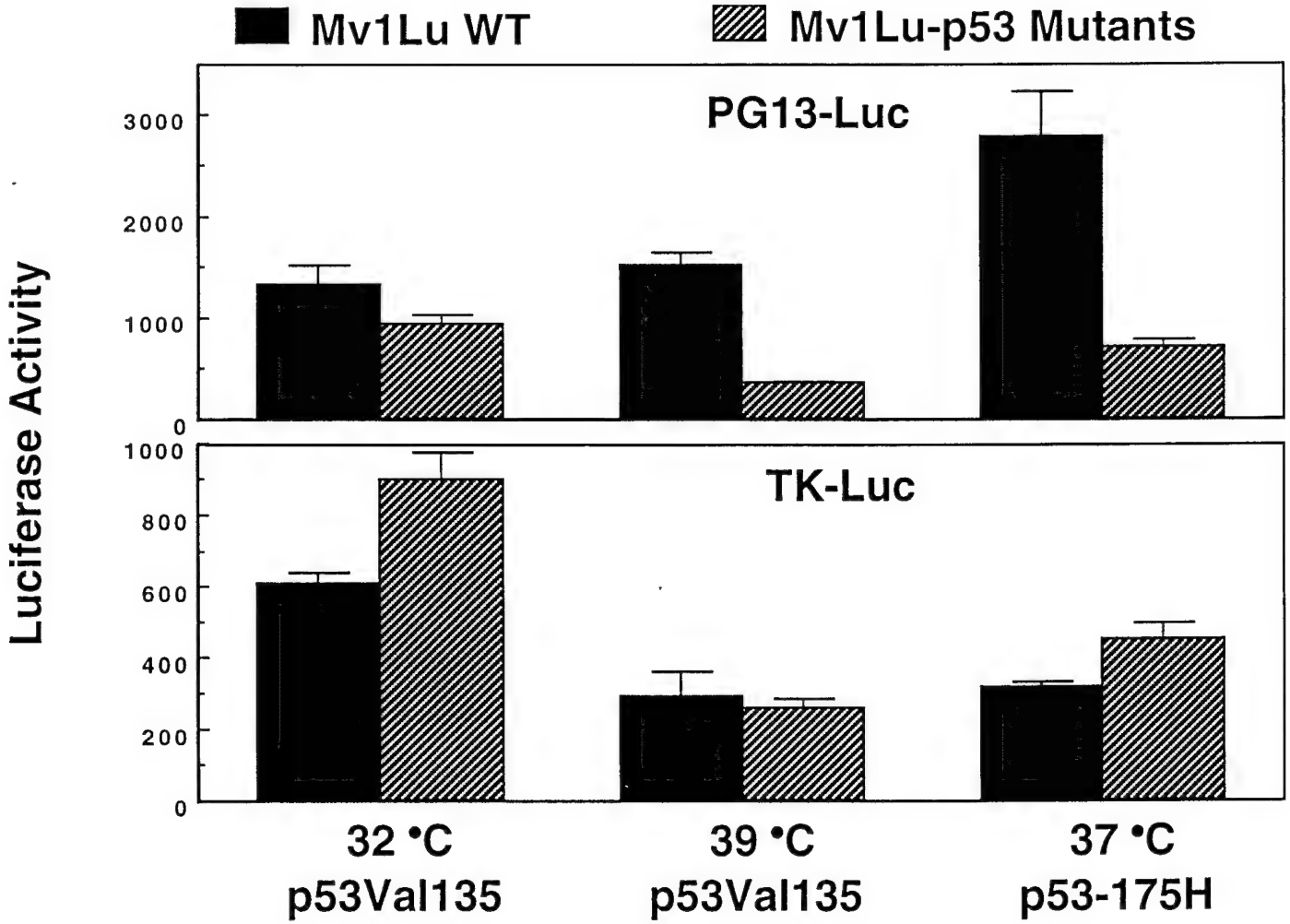

Mv1Lu WT
Mv1Lu-p53 Mutants
PG13-Luc
TK-Luc
Luciferase Activity
3000
2000
1000
0
1000
800
600
400
200
0
32 °C
p53Val135
39 °C
p53Val135
37 °C
p53-175H

Figure 2A

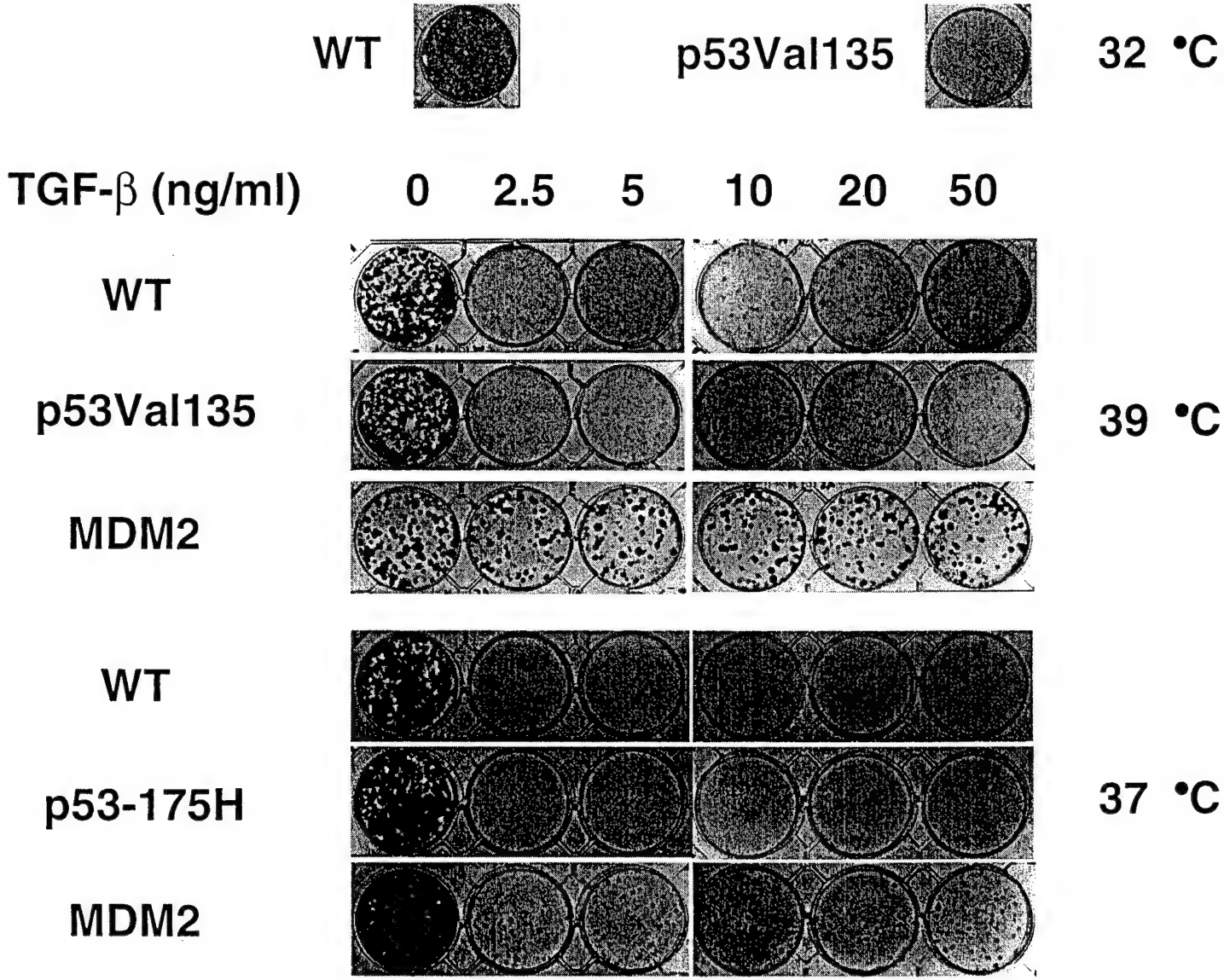
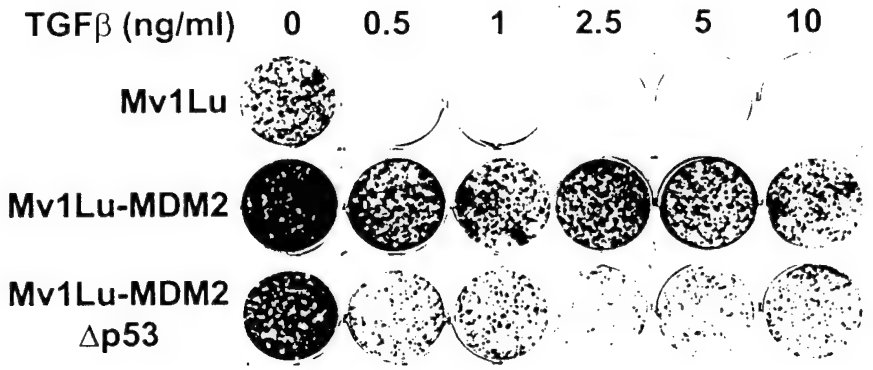

WT
p53Val135
32 °C
TGF-β (ng/ml)
0
2.5
5
10
20
50
WT
p53Val135
MDM2
39 °C
WT
p53-175H
MDM2
37 °C

Figure 2B

C.



D.

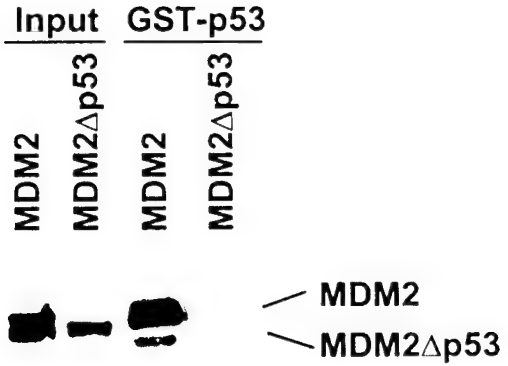


Figure 2C/D

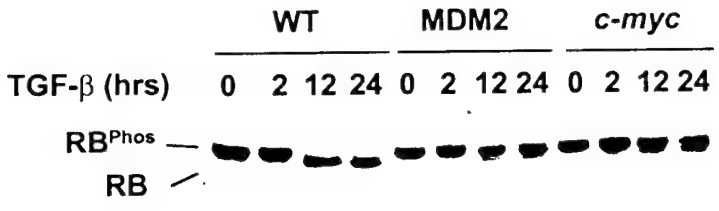


Figure 3A

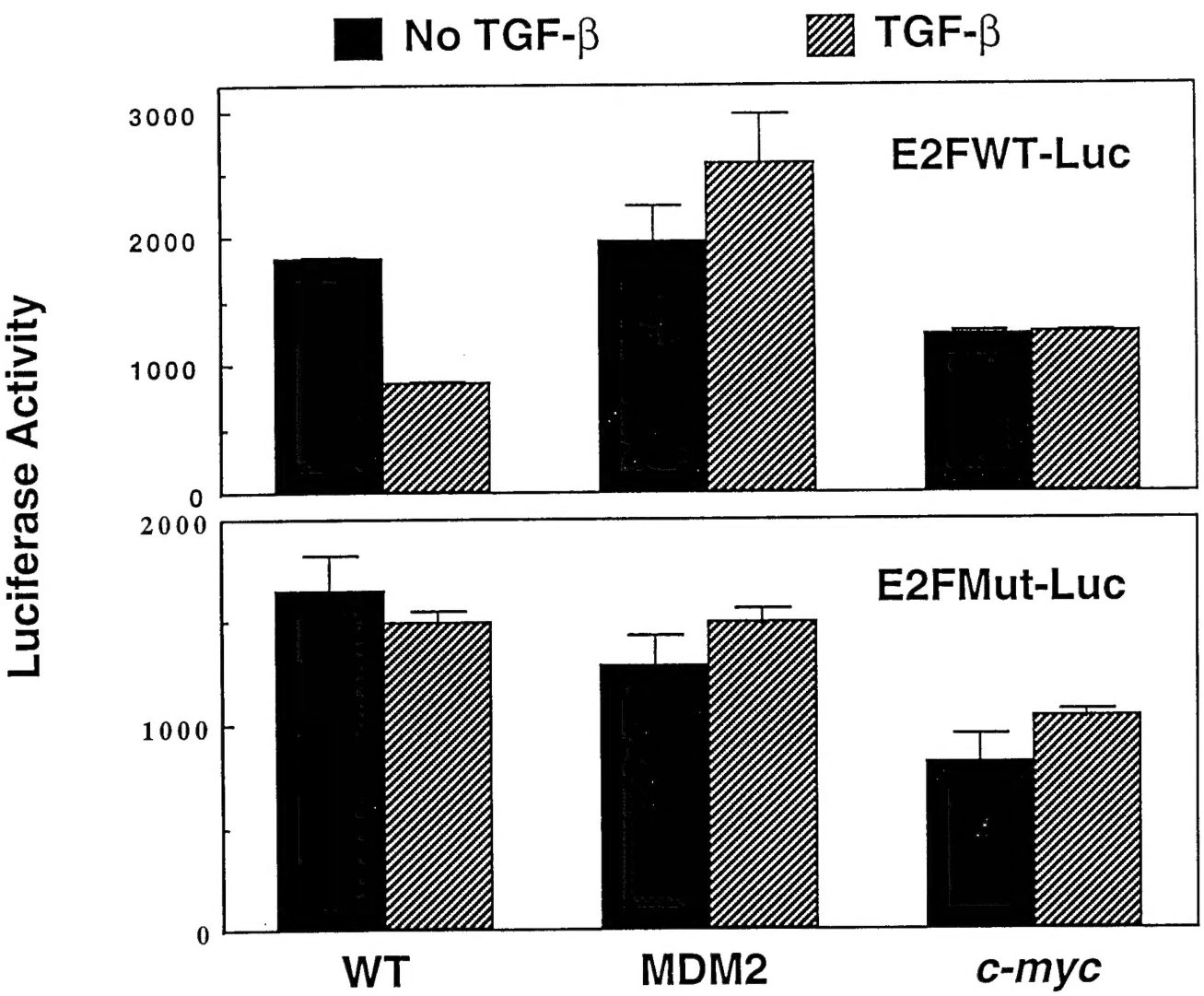

No TGF-β
TGF-β
Luciferase Activity
3000
2000
1000
0
E2FWT-Luc
2000
1000
0
E2FMut-Luc
WT
MDM2
c-myc

Figure 3B

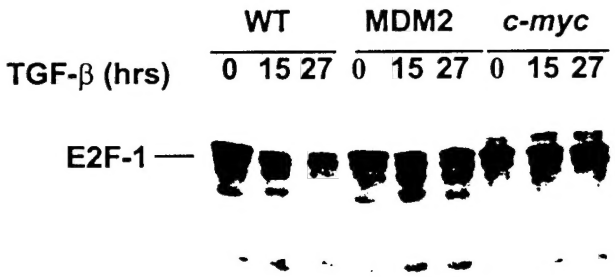


Figure 3C

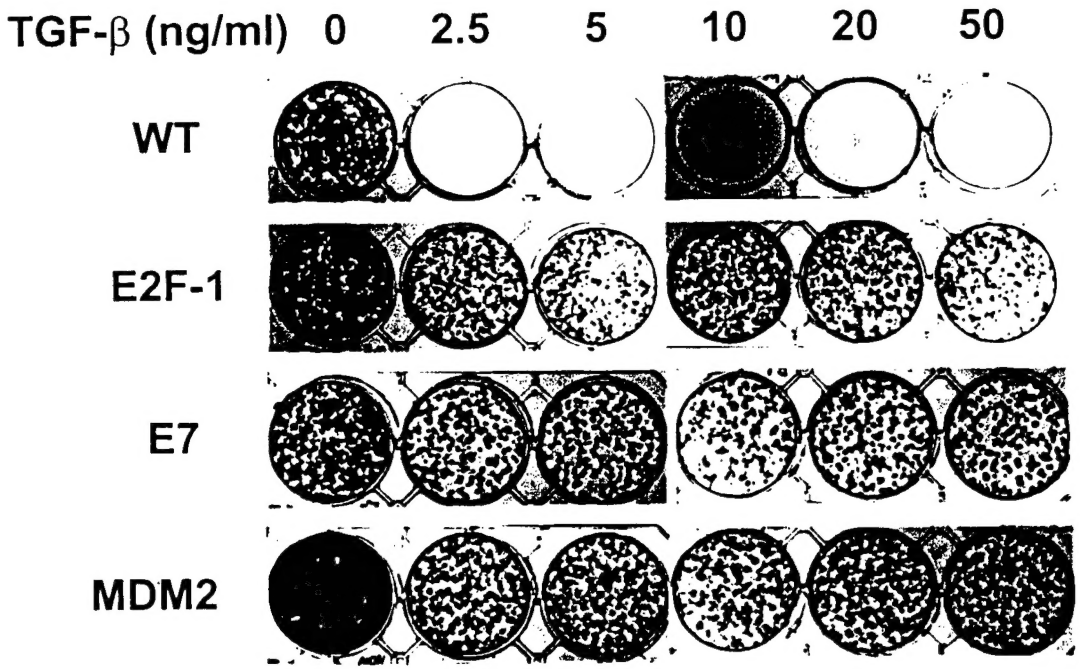


Figure 3D

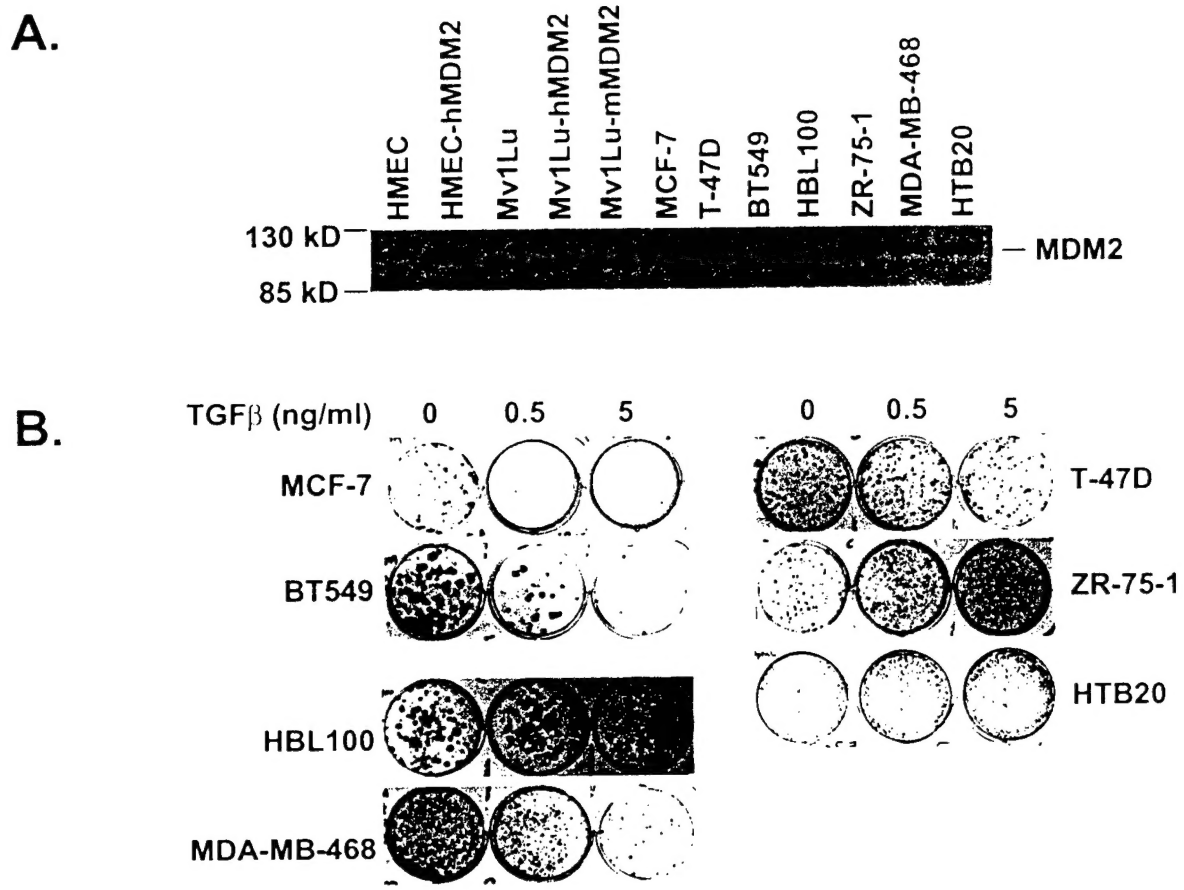

A.
HMEC
HMEC-hMDM2
Mv1Lu
Mv1Lu-hMDM2
Mv1Lu-mMDM2
MCF-7
T-47D
BT549
HBL100
ZR-75-1
MDA-MB-468
HTB20
130 kD
85 kD
MDM2
B.
TGFβ (ng/ml)
0
0.5
5
MCF-7
BT549
HBL100
MDA-MB-468
T-47D
ZR-75-1
HTB20

Figure 4 A B